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Award Number: W81XWH-06-0461

TITLE: A Novel Combination of Thermal Ablation and Heat-Inducible Gene therapy for Breast Cancer Treatment

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CONTRACTING ORGANIZATION: Duke University

REPORT DATE: April 2009

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: (Check one)

☑ Approved for public release; distribution unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
04-30-2009	Annual Summary	31 Mar. 2006 – 31 Mar. 2009

4. TITLE AND SUBTITLE
A Novel Combination of Thermal Ablation and Heat-Inducible Gene Therapy for Breast Cancer Treatment

5a. CONTRACT NUMBER
W81XWH-06-1-0461

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Duke University

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Box 90300, Durham NC 27708

8. PERFORMING ORGANIZATION REPORT NUMBER
Duke University

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Material
Fort Detrick, Maryland 21705-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
High intensity focused ultrasound (HIFU) has been developed as an emerging non-invasive strategy for cancer treatment by thermal ablation of tumor tissue. The feasibility of synergistic combination of HIFU thermal ablation and HIFU-induced gene therapy is interpreted both *in vitro* and *in vivo* using cancer cell lines (HeLa, 4T1 and R3230Ac) transfected with mark gene encoding proteins (GFP and Luciferase) under the control of Hsp70B promoter. Moreover, a transparent cell-embedded hydrogel is developed to allow for simultaneous assessment of lesion formation, gene expression and temperature distribution. This work opens up a new paradigm for synergistic combination of HIFU thermal ablation with heat-induced gene therapy to improve the overall quality and efficacy of breast cancer therapy.

15. SUBJECT TERMS
HIFU, thermal ablation, heat shock response, gene activation

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			19b. TELEPHONE NUMBER *(include area code)*
U	U	U	UU	10	

Table of Contents

INTRODUCTION:

With the advance of modern imaging techniques, the therapeutic application of HIFU has been considerably expanded recently as a non-invasive strategy for cancer therapy [1, 2]. Through HIFU exposure, acoustic energy is focused into a deep-sited tumor volume and converted into heat, which thereafter produces an in-situ cigar-shaped thermal lesion [3]. While the current HIFU can successfully reduce the primary tumor mass, primary tumor recurrence, metastasis and distal tumor growth are still beyond the effective area of HIFU treatment [4]. In light of the fact that hsp70B protein can be dramatically up-regulated due to its response to heat shock stress [5], we hypothesize that a synergistic combination of HIFU thermal ablation and heat-inducible gene expression can be applied to boost both systematic and localized therapeutic effect. This work is to simultaneously investigate tumor ablation and trans-gene expression under the control of hsp70B promoter both *in vitro* and *in vivo*. A cell-embedded tissue-mimicking hydrogel is then developed as a well-controlled platform to correlate lesion formation, gene expression and temperature distribution during HIFU exposure.

BODY:

Image-guided experimental HIFU system:

The HIFU transducer was a single-element air-back annular piezoceramic transducer (H-102, Sonic Concepts, Seattle, WA) operating at its fundamental frequency (1.1 MHz) or its third harmonics (3.3 MHz). The ultrasound imaging system was a 5/7 MHz phased array probe operating with 8-cm maximum imaging depth. A 3D step motor positioning system (Velmex, BisSlide M02, Bloomfield, NY) was employed to align experimental samples with 50-μm resolution and 100-mm traveling range. Operation of the HIFU transducer and the position system were controlled by Labview software interfaced via a GPIB board. Located inside of a Lucite water tank, the HIFU transducer was mounted either at the side-wall for *in vitro* study or on the bottom for *in vivo* study. The ultrasound imaging probe was aligned perpendicular to the HIFU beam at 0^0 or 90^0 orientation.

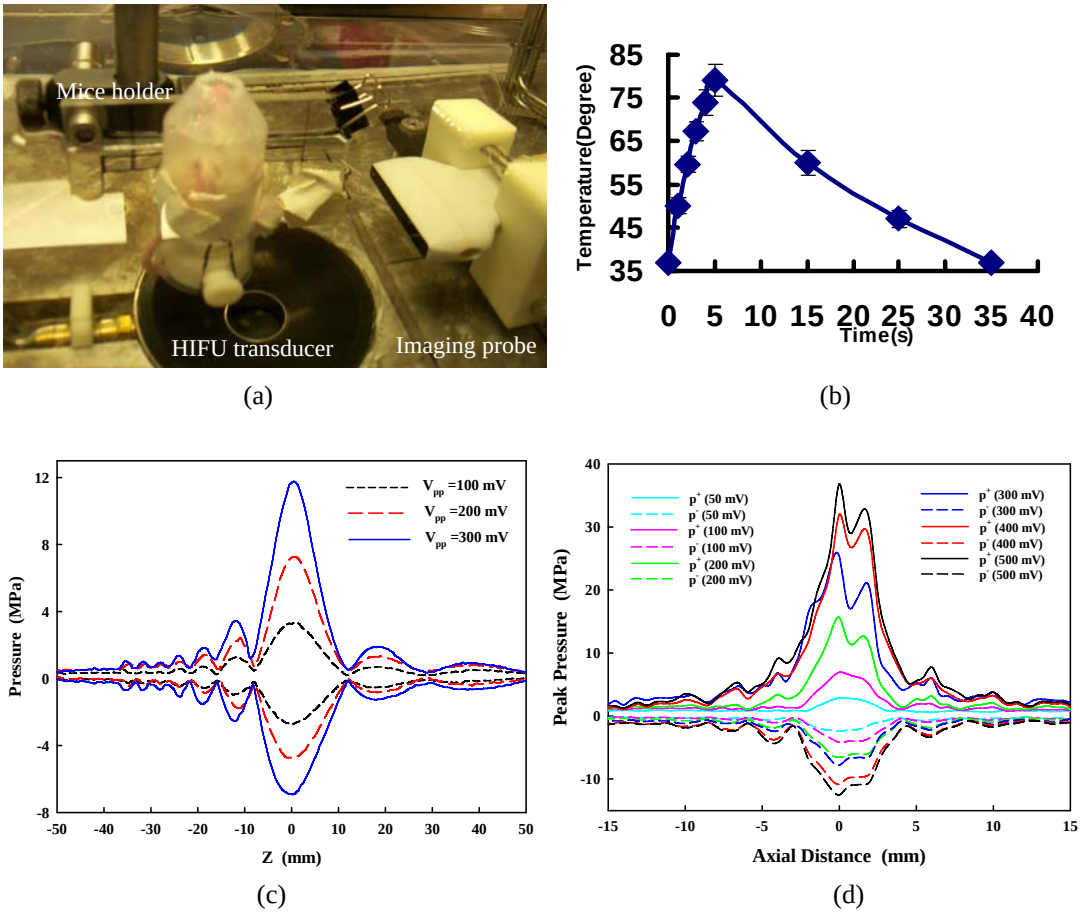


Fig. 1 (a) Experimental HIFU system (b) Representative temperature profile at HIFU focus (c)-(d) Axial pressure distribution at 1.1MHz and 3.3MHz

The temperature increase was recorded by a 0.1-mm bare ware thermocouple (Custom-designed IT-23, Physitemp Inc, Clifton, NJ). The thermal couple output voltage was conditioned by an isothermal terminal block (TC-2190, National Instrument, Austin, TX) and registered by a multiple-channel data acquisition board (NI4351, National Instrument, Austin, TX). A representative temperature elevation was recorded in a porcine liver tissue under 5-s HIFU exposure at intensity level I_{SAPA}=1860 W/cm^2, which assured us the capability of the integrated HIFU system to produce clinical-relevant intensity for coagulative tissue necrosis.

The pressure field around the focal spot of the HIFU transducer was characterized with a fiber optic probe hydrophone (FOPH-500, RP Acoustics, Leutenbach, Germany). The acoustic signals collected by the FOPH were recorded on a digital oscilloscope (LeCroy 9310A, Chestnut Ridge, NY). The pressure distribution in both lateral and axial direction was recorded, and spatial-peak pulse-average intensity (I_{SPPA}) and spatial-average pulse-average intensity (I_{SAPA}) were further calculated [6]. According to the pressure distribution plot, the acoustic energy was shown to concentrate more towards the focus at 3.3-MHz that at 1.1-MHz. As a result, peak pressure and acoustic intensity at 3.3-MHz were much higher than the counterparts at 1.1-MHz, given the same electric output power.

HIFU-Induced Gene Expression *in vitro*:

Rat mammary carcinoma (R3230Ac or Mac) cells were transfected with hsp70B-EGFP plasmid using lipofecfamine (Gibco-BRL, Bethesda, MD). G418 (400 μg/ml) was added to the transfected cell culture and hyperthermia (30-min @ 42℃) was performed to select stably transfected R3230Ac cells. Before HIFU treatment, the transfected R3230Ac cells were trypsinized and pelleted. The HIFU transducer operating at 1.1-MHz was mounted horizontally inside the Lucite water tank filled with degassed water (37℃). A volume of 10-μl cell suspension (5×10^7/ml) was loaded in a 0.2-ml Polymerase Chain Reaction (PCR) tube (#3726, Corning, NY), which was aligned vertically with its bottom within the -6dB HIFU beam focus. The I_{SAPA} was calculated to be 278 W/cm^2 when the function generator was operated at 0.1 V_{pp}. The temperature profile of cell suspension, monitored by 0.1-mm bare-ware thermocouple, was varied by adjusting the duty cycle of the tone burst signal. Immediately after HIFU exposure, cell samples were cultured on the etched-grid cover slips in a 6-well plate for 24-hours. Cell viability was determined by hemocytometer with 5-min *in situ* Trypan blue staining. GFP gene expression was determined by a flow cytometer (FACScan, Becton Dickinson, San Jose, CA). Intracellular hsp70 protein was quantified by Hsp70 ELISA kit (StressGen, Victoria, Canada) with the Bio-Rad protein assay system (Bio-Rad, Hercules, CA, USA).

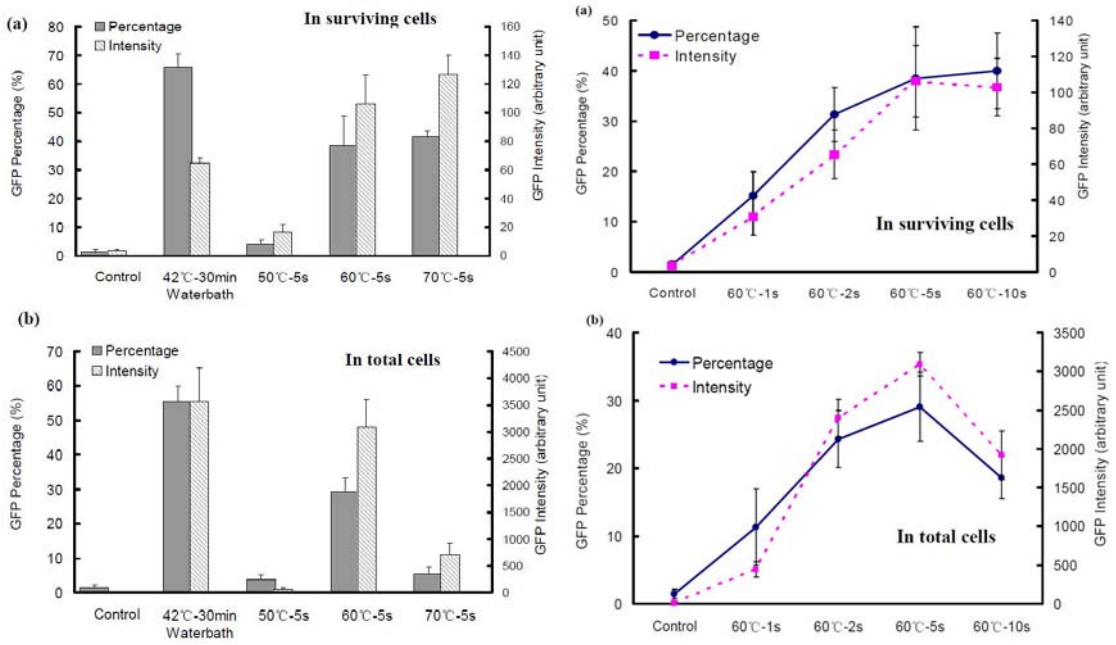


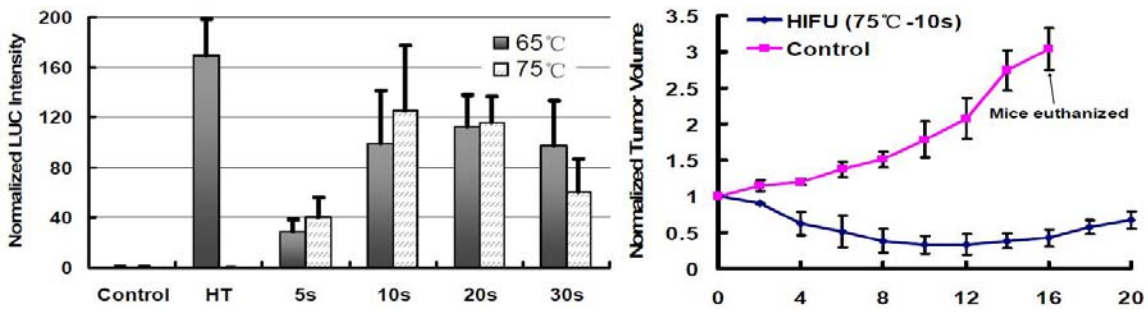
Fig. 2 HIFU-induced GFP expression at different peak temperature (left) and exposure duration (right)

The effect of peak temperature on trans-gene expression in the transfected R3230Ac cells was evaluated at a fixed heat shock exposure time of 5-s by phase contrast and fluorescence microscopy. At 50℃ peak temperature, only 4% GFP expression was induced. As the peak temperature was increased to 60℃ and 70℃, much stronger GFP expression (38% and 41%) was induced in the surviving cells. With respect to the total exposed cells therefore, the maximum gene expression was induced at 60℃ peak temperature, when GFP expression was increased by 20 folds. Comparatively, GFP expression was only increased by 3 folds at 50℃ and 4 folds at 70℃. The effect of treatment duration on trans-gene expression in the transfected R3230Ac cells was evaluated at 60℃ peak temperature. GFP expression in the surviving cells increased initially with time and gradually saturated after 5-s exposure, while

the total GFP expression dramatically dropped after 10-s exposure due to much more cell injury at longer exposure. These findings suggested the most effective gene expression, with respect to the total exposed cells, is not boosted when the cells are exposed to either non-sufficient stress or excessive stress. The optimal window of thermal dosage for inducing the maximum total GFP expression is 5-s at 60℃ in our experimental system. It is also noted, even if the peak temperature was 70℃ or above (data not shown), a small number of tumor cells can still survive, which is comparable to incomplete tumor necrosis by HIFU therapy [7]. In light of this observation, it is desirable to combine HIFU thermal ablation with other therapeutic modalities, e.g. gene therapy, to boost a systematic anti-tumor immune response as well.

HIFU-Induced Gene Expression *in vivo*:

2×10^6 mouse mammary carcinoma cancer cells (4T1) were subcutaneously injected into the right hind limbs of female Balb/C mice (Charles River Laboratory, Wilmington, MA) at 6-8 week old. When the tumor reached 8-mm in diameter, 2×10^8 pfu adenoviral luciferase vectors with hsp70B promoters (Ad-hsp70B-Luc) were injected into the center of the tumor. Virus dissemination and gene transfection inside the tumor took 24 hours before HIFU treatment. In the non-treatment control, intra-tumor injection of Ad-hsp70B was performed with the same protocol without subsequent HIFU exposure. Delivery of HIFU to the target tumor-bearing hind limb was controlled by a B-mode ultrasound imaging-guided system aforementioned. A 0.1-mm bare-wire thermocouple through a butterfly needle was inserted into the center of the tumor to monitor the *in situ* temperature. HIFU exposure was operated in continuous wave (CW) mode at 1.1 MHz. The peak temperature at focus was varied by adjusting the exposure pressure. The time course of Luciferase expression was monitored in a week. On the day of gene expression assay, 100μl aqueous D-luciferin solution was intraperitoneally injected 20-min before *in vivo* analysis with a Xenogen bioluminescence imaging system (Xenogen Inc, Alameda, California). The final images were represented by superimposing the pseudo-color bioluminescence images on conventional grey scale reference images [8]. The tumor size was monitored with an electronic digital caliper every other day for 20 days.



(a) (b)

Fig. 3 (a) Peak Luciferase expression at different exposure duration (b) Time course of tumor growth regression

65℃-75℃ peak temperature has been shown to induce much stronger GFP expression with the earlier *in vitro* experiment of R3230Ac cells. By fixing the peak temperature at 65℃ or 75℃, the effect of treatment duration on trans-gene expression *in vivo* was examined with BLC imaging. Peak luciferase expression was observed 24 hours after HIFU treatment and

decayed gradually to the background level within one week. 10 – 20-s HIFU exposure led to maximum gene activation in the target tumor tissue. Specifically, 1.1 MHz HIFU exposure at 75℃ for 10-s increased Luciferase gene expression by 120 folds. While this elevation was still lower than 168 folds as the output of water-bath hyperthermia, a further justification was performed with an improved HIFU scanning strategy, i.e. 36 spot dense scanning at 3.3-MHz *vs.* the original 5 spot sparse scanning at 1.1-MHz. The Luciferase expression was elevated by 170 folds, which is statistically comparable to the case of water bath hyperthermia. The tumor shrunk to the lowest point of ~33% of the initial size in 12 days, while the control tumor kept increasing to ~300% of the initial size at the endpoint. The tumor recurrence after 12 days is similar to what has been observed after clinical HIFU treatment. This limitation might arise from the fact that part of the tumor cells were only sub-lethally injured and survived HIFU exposure, due to the heterogeneity of tumor tissue [4]. To resolve this problem, HIFU thermal ablation might be synergistically combined with a heat-inducible cytotoxic or immunostimulatory gene therapy. A direct way is to introduce therapeutic gene fragments in the gene construct, e.g. replacing Ad-hsp-Luc with Ad-hsp70B-IL12 or Ad-hsp70B-TNF in this experiment.

HIFU-Induced Gene Expression in a Cell-Embedded Tissue Mimicking Phantom:

The phantom matrix is based on an agarose gel mixed with bovine serum albumin (BSA) and glycerol [9]. R3230Ac cells were stably transfected with GFP under the control of hsp70B promoter in advance. The resulting cell-imbedded tissue-mimicking phantom consists of 2% agarose (A9045, Sigma-Aldrich, St. Louis, MO), 3% (6%, 9%) BSA (A7096, Sigma-Aldrich, St. Louis, MO), and 5% glycerol (G7757, Sigma-Aldrich, St. Louis, MO) by weight, and the R3230Ac cells with concentration from $0.5x10^6$/ml to $5x10^6$/ml. The speed of sound and attenuation were measured with a broadband receiving needle hydrophone using a through-transmission substitution technique [10]. The nonlinear parameter B/A was determined via the finite amplitude inset-substitution method [11]. Thermal conductivity and diffusivity were measured with a thermal property analyzer. The compressive modulus of cylindrical gel phantoms was measured with a rheometer. This constructed phantom has similar sound speed, acoustic impedance, thermal conductivity, thermal diffusivity and compressive modulus to the values in soft tissue, while the attenuation coefficient and nonlinear B/A ratio were comparably lower than those of soft tissue. The attenuation coefficient and the compressive modulus are the only parameters dependant on the BSA concentration. All the physical parameters are independent of cell concentration from $0.5x10^6$/ml to $5x10^6$/ml.

Physical Properties	Attenuation Coefficient (dB cm MHz)	Sound Speed ($m\ s^{-1}$)	Nonlinear Parameter B/A	Compressive Modulus (KPa)	Thermal Conductivity ($W\ m^{-1}\ ℃^{-1}$)	Thermal Diffusivity ($mm^2\ s^{-1}$)
Soft Tissue	0.5	1540	6.8-8.5	10-120	0.47-0.6	0.12-0.15
Phantom	0.17	1500	5.5	4.7-8.7	0.56	0.13

Table 1 Physical property of clel-embedded ultrasound phantom (20℃)

Thermal lesions were produced in a gel phantom (with 6% BSA) at the focal point of a 3.3-MHz HIFU transducer. Temperature distribution was recorded by an embedded thermo-

couple array consisting of 5 bare-wire T-type thermocouples of 0.1 mm diameter. After HIFU treatment, the gel phantom was sectioned into ~1.5-mm thin slices along the direction parallel to the focal plane and cultured for another 24-h before visualization of GFP activation by fluorescence microscopy. Cigar or tadpole shape thermal lesions were observed inside the gel phantom, dependent on different combinations of ultrasound intensity and exposure duration. Typical lesion size ranged from 1×3 mm to 7.5×15 mm. One day following the 10-s HIFU exposure, the white thermal lesion spot remained visible under phase contrast microscope. At this stage, GFP positive cells were primarily observed within a circular band in the focal plane. The *in situ* equivalent thermal dose EM_{43} in this plane was calculated based on the thermocouple array measurement. The correlated peak temperature for the gene activation zone after 10s HIFU exposure ranged from 54℃ to 63℃. It is found that the empirical thermal necrosis boundary (EM_{43} =240min) fell into gene activation ring in both focal and beam planes, suggesting that gene activation was primarily induced in the sub-lethally injured cell population outside the HIFU lesion boundary.

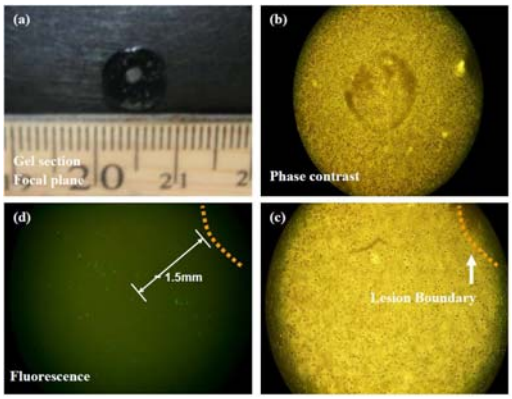


Fig. 4 (a) Gel section (b) Lesion view (c) Lesion boundary (d) GFP expression

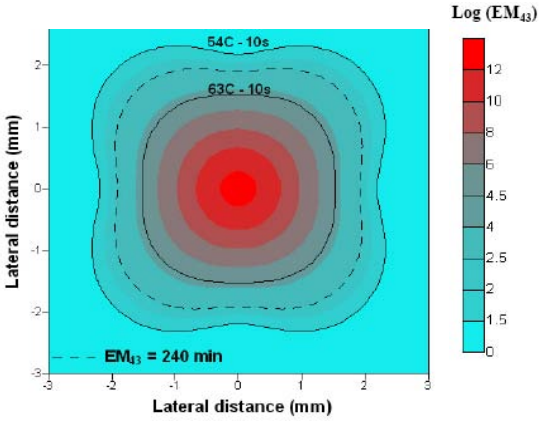


Fig. 5 EM_{43} within focal plane

KEY RESEARCH ACCOMPLISHMENTS:

- ✧ Integrated and characterized an image-guided experimental HIFU exposure system
- ✧ Investigated HIFU induced thermal dose dependent GFP expression in R3230Ac cell suspension
- ✧ Investigated simultaneously tumor ablation and HIFU induced thermal dose dependent Luciferase expression with a tumor-bearing murine model
- ✧ Developed and characterized a 3D cell-embedded tissue mimicking phantom and utilized this phantom to correlate localized GFP expression with *in situ* delivered thermal dosage

REPORTBAL OUTCOMES:

1. Liu Y, Zhong P. High intensity focused ultrasound induced trans-gene activation in a cell-embedded tissue mimicking phantome. *IEEE ultrasounics Symposium*, Vancouver, Canada, 2006

2. Liu Y, Zhong P. Development of a cell-embedded tissue mimicking ultrasound phantom. *Acoutical Society of American 151st meeting*, Providence, RI, 2006

3. Yuan F, Pua C, Liu Y, Zhong P. HIFU-Induced Gene Activation in a Cell-Embedded Tissue Mimicking Phantom. *International Mechanical Engineering Congress and Exposition*, Seattle, WA, 2007

4. Yuan F. Pua C, Liu Y, Zhong P. A Novel Combination of Thermal Ablation and Heat-Inducible Gene Therapy for Breast Cancer Treatment. *DOD BCRP Era of Hope meeting,* Baltimore, MA, 2008

CONCLUSION:

In this work, we have demonstrated that HIFU at thermal ablation level can induce trans-gene expression under the control of hsp70B promoter both *in vitro* and *in vivo*. An image-guided experimental HIFU system was established to spatially and temporally regulate the process of HIFU treatment. The gene expression activity was identified to be dependent on the *in situ* delivered thermal dosage, determined by the interplay of peak temperature, treatment duration and treatment strategy. The underlying mechanism of HIFU-induced gene expression was further investigated by correlating the gene expression pattern with thermal dose distribution around a HIFU-induced thermal lesion in a new developed cell-embedded tissue-mimicking phantom. Maximum gene expression was suggested to be produced as the delivered HIFU thermal dosage was just enough to employ sub-lethal damages on tumor cells. Since cancer recurrence observed following clinical HIFU therapy may arise from the sub-lethally injured tumor cells that survive the HIFU treatment, the capability to regulate the pathway of these sub-lethally injured cells in a cancer curing favorable direction can dramatically improve the current HIFU therapy. Conceptually, heat-inducible cytotoxic (TNF-α) or immunostimulatory (IL-12) genes can be incorporated to the target tumor tissue, and these genes will be greatly activated, according to our findings, in sub-lethally injured tumor cells during HIFU thermal ablation, to eradiate residual or distal metastasis tumor cells [12]. To expand the current work into clinical application of breast cancer treatment, the biological consequence of sub-lethally injured cancer cells, including proliferation, cell lyses, apoptosis and intracellular HSP protein levels, have to be quantified and compared under different HIFU exposure dosages. A new generation of cell-embedded tissue-mimicking phantom can be developed with an even closer physical and biological property to human tissue, and a better physiological environment to maintain cell phenotype and activity. In conclusion, the present work opens up a new paradigm for synergistic combination of HIFU thermal ablation with heat-induced gene therapy to potentially improve the overall quality and efficacy of breast cancer therapy.

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